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THE VIABILITY OF THE WINTER SPORANGIUM OF *SYNCHYTRIUM ENDOBIOTICUM* (SCHILB.) PERC., THE ORGANISM CAUSING WART DISEASE IN POTATO

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(With 1 Diagram.)

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I. A STAINING METHOD FOR TESTING THE VIABILITY OF THE WINTER SPORANGIUM.

Introduction.

In attempting to eradicate *Synchytrium endobioticum* from contaminated soil the most rational and economical sequence of study is to determine what conditions bring about death of the causal organisms *in vitro* and to reproduce these conditions first on a pot scale and afterwards in the field. This systematic method of attack has not been possible because no technique was known for testing *in vitro* the viability of the organism and because methods of pot experiment proved unsatisfactory. Field experiments though slow and costly have therefore been used to a large

extent independently of work in the laboratory and glasshouse. Extensive field tests have been carried out by a number of workers including Malthouse (7), Schaffnit and Voss (10) and members of this institution (9), but no satisfactory and economic means of sterilising soil contaminated with *Synchytrium endobioticum* has been found. Further, field results are difficult to interpret in that plots treated in the same way often give different results in different years. Precise methods are therefore needed so that fundamental knowledge regarding the viability of the organism causing Wart Disease may be obtained.

Pot experiments have been used to a limited extent in the study of Wart Disease, but were found unreliable in that infection even of the controls appeared erratic. A study was therefore made of the factors controlling infection of potato plants grown in pots and it was shown that under suitable conditions, one of which is a very high degree of soil moisture during part of the growing period, a high percentage of infected plants could be obtained regularly in small pots (7-inch diameter) (3). This partial standardisation of the method made it possible to compare the results of infection experiments carried out in pots with other means for testing viability of the Wart Disease organism.

A simple technique for testing *in vitro* the viability of the winter sporangium of *Synchytrium endobioticum* has been sought. The standard method of comparing the germination of samples of spores after subjection to various treatments has not been feasible because of the great difficulty experienced in inducing winter sporangia to germinate under laboratory conditions. Curtis (1) and others have with difficulty obtained a small percentage germination of winter sporangia kept in water for about ten weeks, and have actually observed the escape of zoospores, but such methods of germination are too uncertain and uncontrollable to provide a convenient technique for testing viability.

In the absence of a method for observing the actual germination of winter sporangia an estimate of the numbers of sporangia germinating in a given time might be made by counting the numbers of empty sporangia left. This has been attempted but the method is open to several objections. In the first place increase in the proportion of empty sporangia is slow. Counts made of the numbers of empty sporangia in three sets of one hundred removed at intervals from a tube containing sporangia in tap water at 18° C. are given in Table I.

The percentage of empty sporangia in a sample is small even after 13 weeks and the variation in different counts made at the same time is considerable. Direct evidence is needed that the presence of empty

Table I.

Percentage of Empty Sporangia developing in tap water at 18° C.

Date of Experiment: 5. vii. 21–6. x. 21.

	Original sample	After 3 weeks	After 6 weeks	After 13 weeks
Number of empty sporangia per hundred counted	4	14	9	14
	5	12	10	12
	0	9	12	21
Average percentage of empty sporangia	3	11·6	10·3	15·6

sporangia is due to germination and not to degeneration of the cell contents or to the activity of parasites which have recently been described by Köhler (4). Again fracture by mechanical means is very easily effected and produces empty sporangia resembling those which have germinated. Although it might be possible to develop a method of testing viability by observing the increase in numbers of empty sporangia it appeared likely to be tedious and unsatisfactory. A more rapid if less direct method was therefore sought.

The present communication describes experiments in which the reaction to certain stains is compared and correlated with the infecting power of sporangia which have been subjected to various treatments. Assuming that infecting power is a direct test of viability it is shown that there is a clear relationship between certain staining reactions of the sporangia and their viability, so that a rapid means of testing the latter becomes available.

PRESENT INVESTIGATION.

Staining Reactions of Sporangia.

To find a method for testing the viability of winter sporangia of *Synchytrium endobioticum* it was necessary to compare the reactions of dead with those of living sporangia. Large numbers of winter sporangia fairly free from other organic matter were obtained by pressing decaying warts through a finely woven fabric mesh ($210\mu \times 210\mu$) and then centrifuging about twenty times. Of these sporangia, some were not treated, others were boiled for a few minutes in water and another sample was treated with commercial formaldehyde for two hours and then washed free from the reagent. The assumption was made that the untreated sporangia were alive and that the sporangia subjected to each of the drastic treatments described were dead.

The reactions of treated and untreated sporangia to a number of

stains, such as safranin, eosin, methylene blue, orange G, methyl violet, acid fuchsin etc. all in aqueous solutions, were observed. The thick brown sporangial wall not only hinders penetration but also makes it very difficult to observe the effect of the stain on the cell contents. By gently pressing down the cover-slip the sporangial walls were broken and the contents expressed into the stain while under observation.

The most decisive difference in the response of treated and untreated sporangia to the stains tested was found in the case of acid fuchsin 1–2 per cent. aq. The contents of untreated sporangia when pressed out into the stain slowly assumed a pale pink colour which gradually deepened. The expressed protoplasm generally showed considerable Brownian movement and its appearance suggested liquidity and mobility. In the case of treated sporangia the cell contents rapidly stained deep crimson and appeared coagulated, Brownian movement being absent. This difference in reaction was very striking and was observed in treated and untreated sporangia obtained from warts of very different ages. Further a number of samples of sporangia treated with different killing and fixing reagents for 24 hours and then washed, all stained rapidly and deeply when pressed out in acid fuchsin.

It therefore appeared possible that the acid fuchsin reaction of sporangia might be an indication of their viability. Extensive tests were carried out to discover what correlation could be found between the reaction of sporangia to acid fuchsin and their infecting power. Winter sporangia were subjected to a number of treatments varying greatly in intensity and type. These included subjection to various temperatures, and treatment with diverse chemicals such as a strong and a weak acid, a strong and a weak base, and various sterilising agents such as formaldehyde and phenol. Treated sporangia were examined by the acid fuchsin test and others similarly treated were used to inoculate potato plants grown in pots. The results were then compared.

Technique.

A measured quantity, 60 c.c., of dry warted material which had been pressed through a 3 mm. sieve was used for each treatment in the earlier experiments, *i.e.* the temperature series and sulphuric acid Set I. For sulphuric acid Set II and for subsequent experiments a much smaller quantity of sporangia was prepared by pressing wet warts through muslin, allowing to settle and pouring off the water. About 5–10 c.c. of the damp sporangial matter was used for each treatment. This was more easily wetted and convenient to use than the larger quantity though

it is liable to give slightly less infection. For each treatment the sporangial material was wetted as thoroughly as possible with 300 c.c. or when small quantities of sporangia were used with 200 c.c., using water in the temperature set and an aqueous solution of the particular chemical in the chemically treated series. After the chemical treatments the sporangia were freed from the reagent by repeated aqueous extractions followed by washing on fine silk spread over a filter funnel.

A. *Staining Test with Acid Fuchsin.* Treated sporangia separated from other organic material by pressing through fine voile and centrifuging were examined in acid fuchsin 2 per cent. aq. The coverglass was gently pressed so that the sporangial wall was broken and the contents expressed into the stain. At least 20 sporangia per test were examined in this way and their reaction noted. Sporangia which behaved in the same way as the controls were described as reacting with a "faint" stain. The intermediate group containing some faintly and some deeply staining sporangia together with some intermediate in reaction were classed as "intermediate," the relative proportions of each type varying considerably after different treatments. The third group consisted of sporangia which all stained deeply and rapidly.

B. *Infection Experiments in Pots.* When the larger quantity, 60 c.c., of sporangial material was used about five-sixths of it was mixed with each 15 lbs. of soil and the rest put directly on the tubers as they were planted. When the smaller quantity of sporangia was used it was all put on and around the tubers.

The soil consisted of a mixture of heavy soil from Rothamsted fields, sand and an organic material consisting of rotted cavings in the following proportions: soil 37·5 per cent., sand 50 per cent., cavings, 12·5 per cent. Each 15 lbs. of soil was divided and put into 5-inch earthenware pots containing sand to a height of about 1 inch, each standing in a saucer. A single tuber of the very susceptible variety Arran Chief was planted in each pot. The soil was kept fairly dry for two or three weeks until the shoots appeared above the surface, after which the soil was kept extremely wet with water standing almost continually in the saucers. Watering was effected by pouring water into the saucers and not directly on to the soil.

The potato plants were examined for Wart Disease after three months. In some cases the permanency of the effect of treatment on sporangia was tested by growing a second crop in the same soil after the completion of the first infection experiment. The soil from each set of five pots containing similarly treated sporangia was put in one large pot in

which three tubers were planted, watering being carried on as before. After three months the presence or absence of infection was noted.

Effect of Various Treatments on Staining Reaction and Infection Power of Winter Sporangia.

A. *Temperature.* The sporangial material in water was put on a water bath and raised to the required temperature at which it was maintained for one hour. The time taken to reach these temperatures varied from about 10 to 30 mins. Two sets of experiments were run, the second set repeating treatments at certain temperatures but using double the quantity of sporangial material so that twice the number of plants could be infected. In some cases a second crop was grown in the same soil after the completion of the first experiment.

Table II.

Effect of Temperature on the Staining Reaction and Infecting Power of Sporangia treated in Water.

Time: 1 hour.

Temp. ° C.	Stain in acid fuchsin	Infection in pot experiments: 1st crop: Number of plants	Number of plants infected	Percentage of plants infected	Infection in 2nd crop grown in same soil
Room temp.	Faint	9*	8	97	Present
		20	20		
30	,,	4	4	100	,,
40	,,	4	4	100	,,
45	,,	4	3	86	,,
		10	9		
50	Intermediate, majority faint	4	2	79	,,
		10	9		
55	Intermediate	2	0	8	,,
		10	1		
60	Intermediate, majority deep	4	0	5	,,
		15	1		
70	Deep	2	0	0	Absent
		5	0	0	,,
80	,,	5	0	0	,,
		5	0	0	,,

* In the first set some plants failed to grow because they were kept too wet before the sprouts had appeared above the surface of the soil. In later experiments this was remedied and very few failures were obtained.

Table II shows that when the sporangia stained faintly after treatment there was no apparent reduction in infection, when their reaction was intermediate a reduction in infection was apparent, while after

treatments which produced deeply staining sporangia no infection occurred. The permanency of the treatment was indicated by similar infection in the second crop.

B. *Sulphuric and Acetic Acids, Formaldehyde, Phenol, Ethyl Alcohol and Chloroform.* Winter sporangia were treated at room temperature with various concentrations of sulphuric acid for periods varying from 24 hours to 17 days. Two sets of infection experiments differing in the quantity of infecting material used, were put up side by side. In Set I infection was carried out as usual by using 60 c.c. of sporangial material, part of which was mixed with the soil and part applied directly on to the tuber while in Set II about 5 c.c. of sporangial material was used and this was all put directly on to the shoots of the tubers.

Tests were later carried out with acetic acid, formaldehyde, phenol, ethyl alcohol and chloroform, using the smaller quantity of sporangial material as in Set II of the sulphuric acid series.

The results are collected together in Table III which shows that in the sulphuric acid series infection was just as great whether the smaller or larger quantity of sporangial material was applied. The smaller amount was therefore used in subsequent experiments.

The relation between the acid fuchsin reaction of sporangia and their apparent infecting power was the same after each treatment as it was after subjection to various temperatures. Treatments after which sporangia stained faintly, as did the controls, did not appear to alter the infecting power of the sporangia, while intermediate treatments tended to reduce it and stronger treatments which affected sporangia so that their contents stained deeply and rapidly in acid fuchsin destroyed their infecting power altogether. As the latter may be regarded as a measure of sporangial viability a clear relationship is evident between the reaction to acid fuchsin and the viability of sporangia.

The results obtained in the second crop grown in one soil were similar to the first which favours the view that the effect on the sporangia was permanent.

Acetic acid proved less toxic than sulphuric acid which is worthy of particular note in view of its greater penetrating power and of the opposite results obtained by Loeb (6) experimenting with sea-urchin eggs.

C. *Alkaline Reagents.* Acid fuchsin is a disulphonic acid whose neutral salts are colourless while the acid itself and its mono-acid salts are red. It seemed probable therefore that if the contents of sporangia killed by the action of an alkaline reagent were expressed into acid fuchsin the neutral colourless salt would be formed and the sporangial

Table III.

The Effect of Various Chemical Reagents on the Reaction of Sporangia to acid fuchsin and on their infecting power.

Room Temperature.

Treatment	Concentration	Duration of treatment	Stain in 2 % acid fuchsin aq.	Infection in pot experiments: Set I, 1st crop: No. of plants	Set I, 1st crop: No. of plants infected	Set I: 2nd crop	Set II, 1st crop: No. of plants	Set II, 1st crop: No. of plants infected	Set II: 2nd crop	Percentage infection in 1st crop
Control	—	—	Faint	15	15	Present	10	10	Present	100
Sulphuric acid	*N*/500	24 hours	,,	5	4	—	5	5	,,	90
	N/500	7 days	,,	5	4	Present	—	—	—	80
	N/100	24 hours	,,	5	5	,,	5	5	Present	100
	N/100	7 days	,,	5	5	—	—	—	—	100
	N/10	24 hours	,,	5	4	Present	5	5	Present	90
	N/10	7 days	,,	5	5	,,	—	—	—	100
	N/5	24 hours	Intermediate	5	5	,,	5	5	Present	100
	N/5	3 days	,,	5	3	,,	—	—	—	60
	N	24 hours	Deep	5	0	Absent	5	0	Absent	0
	5 *N*	24 ,,	,,	5	0	,,	5	0	,,	0
Control	—	—	Faint	—	—	—	10	8	—	80
Acetic acid	*N*/500	24 hours	,,	—	—	—	5	5	—	100
	N/250	24 ,,	,,	—	—	—	5	4	—	80
	N/100	24 ,,	,,	—	—	—	5	5	—	100
	N/50	24 ,,	,,	—	—	—	5	5	—	100
	N/10	24 ,,	,,	—	—	—	5	5	—	100
	N/5	24 ,,	Intermediate	—	—	—	5	1	—	20
	N	24 ,,	,,	—	—	—	5	1	—	20
	5 *N*	24 ,,	Deep	—	—	—	5	0	Absent	0
Control	—	—	Faint	—	—	—	10	9	—	90
Formaldehyde	0·5 %	24 hours	,,	—	—	—	5	5	—	100
	1·0 %	24 ,,	Intermediate	—	—	—	5	0	—	0
	5·0 %	24 ,,	Deep	—	—	—	5	0	—	0
	10·0 %	24 ,,	,,	—	—	—	5	0	—	0
Control	—	—	Faint	—	—	—	15	12	—	80
Phenol	*M*/100	24 hours	,,	—	—	—	5	4	—	80
	M/10	24 ,,	Intermediate	—	—	—	5	0	—	0
	M	24 ,,	Deep	—	—	—	5	0	—	0
Control	—	—	Faint	—	—	—	10	9	—	90
Ethyl alcohol	100 %	1 hour	,,	—	—	—	5	5	—	100
	—	24 hours	Intermediate	—	—	—	5	1	—	20
	—	96 ,,	Deep	—	—	—	5	0	—	0
Chloroform	100 %	1 hour	Faint	—	—	—	4	4	—	100

Table IV.

The Effect of Alkaline Reagents on the Staining Reaction and Infecting Power of Sporangia.

Time 24 hours. Room temperature.

Treatment	Concentration	Appearance of sporangial contents in water	Stain in 2 % acid fuchsin aq.	Stain in 2·5 % methylene blue aq.	No. of plants	No. of plants infected	Percentage of plants infected
Sodium hydroxide	Control	Granular with rapid movement	Faint	Faint	15	10	67
	N/100	,, ,,	,,	,,	5	3	60
	N/10	,, ,,	,,	,,	5	2	40
	N	Translucent coagulated	Absent	Deep	5	0	0
	5 *N*	,, ,,	,,	,,	5	0	0
Ammonium hydroxide	Control	Granular with rapid movement	Faint	Faint	15	10	67
	N/100	,, ,,	,,	,,	5	3	60
	N	,, ,,	Intermediate	Intermediate	5	1	10
	5 *N*	Majority apparently solid, some liquid	,,	,,	5	1	10
	Sp. gr. 0·88	Translucent coagulated	Absent	Deep	5	0	0

contents would therefore remain colourless. Deep staining of the sporangial contents killed by an alkaline reagent might however take place in a basic dye. This possibility was tested with sporangia treated with the strong base, sodium hydroxide, and the weak base, ammonium hydroxide. Staining tests were carried out both in acid fuchsin and in the basic dye methylene blue. The appearance of the sporangia in water was also noted.

The contents of untreated sporangia and those treated with less concentrated hydroxides stained faintly in acid fuchsin and again in methylene blue while in water their contents appeared granular and showed considerable Brownian movement. Such sporangia produced infection in potato plants grown in pots.

After treatment with the more concentrated hydroxides the sporangial contents remained colourless in acid fuchsin but stained rapidly and deeply in methylene blue. These sporangia differed from the unaffected sporangia in that their contents had become clear and translucent, which could be observed without breaking the sporangial wall. This effect was never observed after any of the treatments previously described. When expressed into water the sporangial contents appeared coagulated and showed no Brownian movement. Treatments which caused sporangia to behave in this way completely destroyed their infecting power. Treatments producing an intermediate reaction tended to reduce infection.

It therefore appears that an indication of the viability of sporangia treated with strongly alkaline reagents can be obtained both from the appearance of their contents in water and from their reaction to methylene blue.

CONCLUSIONS.

The viability of the winter sporangium of the fungus *Synchytrium endobioticum* can be determined by means of staining tests. Sporangia which have been treated thermally or by various concentrations of reagents which are not alkaline in reaction, can by means of the acid fuchsin test be divided into three groups. Sporangia which, like the controls, stain faintly and slowly in acid fuchsin: these give a high percentage infection in potato plants grown in pots and therefore are alive. Groups of sporangia which are intermediate in reaction, some staining faintly, some deeply and rapidly, and some being intermediate; these tend to cause reduced infection indicating, that while some of them are alive others are probably dead. Finally groups of sporangia which all

stain deeply and rapidly in acid fuchsin: these produce no infection, which is presumptive evidence of death.

Sporangia which have been treated with alkaline reagents do not respond to acid fuchsin but this may be replaced by the basic stain methylene blue in which living sporangia stain faintly and dead ones deeply and rapidly. Further the appearance of the sporangial contents after treatment with alkaline hydroxides is an indication of their viability; the contents of living sporangia appear normal while dead ones are clear and translucent.

II. A METHOD FOR EXTRACTING WINTER SPORANGIA FROM SOIL.

The value of any method for testing the viability of the winter sporangium of *Synchytrium endobioticum* is likely to be greatly increased if it can be applied to sporangia which have been treated actually in soil. A simple method for extracting sporangia from soil, without affecting their viability, was therefore sought. A mechanical analysis of a sample of soil heavily contaminated with winter sporangia was carried out by the water settling method, with the idea that if the sporangia were sufficiently similar to each other in size and specific gravity they would be concentrated in one fraction of the soil. Most of the sporangia were recovered in the silt and fine silt but the remainder were scattered to a considerable extent through the other portions. The concentration of sporangia in any one fraction did not appear great enough to provide a method for recovering a sufficient number of sporangia to carry out viability tests, although Schander and Richter(11) have used a somewhat similar method for recognising contamination of soil by Wart Disease. A more rapid, efficient and less laborious method seemed desirable.

The probable difference in specific gravity of soil and winter sporangia appeared likely to provide the basis for a method of separation. An estimate was therefore made of the average specific gravity of the winter sporangium.

This was calculated by Stokes' formula as used in the mechanical analysis of soil(2) after estimating the rate of fall through water and the diameter of the average sporangium. To determine the rate of fall of sporangia in water a tube about 6 inches long, filled with water, was placed in a vertical position. Sporangia, first carefully separated from each other and from plant tissues by sieving through voile and centrifuging, were introduced in drops of water carefully placed on the surface of the water in the tube by means of a Pasteur pipette with as little disturbance as possible. The sporangia, after they had fallen a distance of about

4 cm. and apparently had assumed a steady rate of fall, came into the field of vision of a low power travelling-microscope placed horizonally and adjusted to observe the fall of the sporangium through 1 cm. The times in seconds taken by 18 sporangia chosen at random to fall the distance of 1 cm. in water were as follows: 37, 38, 6, 15, 20, 39, 57, 30, 30, 35, 22, 45, 74, 18, 23, 25, 51 and 69 giving an average of 35 secs. with a probable error of $\pm$ 4·3. The diameters in micro-millimetres of 12 sporangia also chosen at random were 55·2, 68·6, 46·0, 50·0, 43·0, 64·4, 40·0, 63·0, 50·0, 55·4, 44·2 and 56·6 giving an average of 56 μ with a probable error of approximately $\pm$ 2 μ. The average specific gravity calculated from these figures is 1·175 with a probable error of $\pm$ 0·021.

The specific gravity of ordinary arable soil as given by Warington (13) is 2·5 while Mosier and Gustafson (8) found it varied from 2·57 to 2·65 in estimations made on several different kinds of soil.

The difference in specific gravity of soil and sporangia appeared sufficiently great to allow of their separation by means of some liquid of intermediate specific gravity which should also have a low viscosity and should not affect the viability of the sporangium. Tetrachlorethane (sp. gr. 1·6) and chloroform (sp. gr. 1·5 approx.) both have a low viscosity and were tested for this purpose. The former, although not unpromising, gives off vapours which are very toxic to human beings, so that chloroform was preferred. Air-dry soil passed through a 3 mm. sieve was heavily contaminated with sporangia, and passed through voile with a mesh of 210 $\times$ 210 μ. The sporangia passed through while larger pieces of organic matter were left behind. About 20 gm. of this soil were then shaken up with chloroform and allowed to stand until the soil had settled at the bottom. A separation was immediately made into three portions which consisted of:

(*a*) Particles floating on top of the chloroform. These were mostly sporangia together with small pieces of organic matter.

(*b*) Particles actually in the chloroform which were filtered off. Large numbers of sporangia together with traces of organic matter were found in this portion.

(*c*) The residue consisting chiefly of soil with which very few sporangia had settled.

A second extraction of the residue gave a few sporangia in the chloroform but far the greater quantity were obtained in the first extraction.

The method consists therefore of careful sieving of contaminated soil, to reduce the size and quantity of organic matter which will be separated

with the sporangia, followed by extraction with chloroform. The whole of the chloroform is pipetted off as soon as the soil has settled and is then filtered. The filtrate is examined for sporangia.

Sporangia separated in this way and examined by the acid fuchsin staining test showed no effect of the chloroform on the viability of the sporangium. Table III shows that sporangia treated with chloroform for one hour still stained faintly and slowly as did untreated sporangia, and that their infecting power appeared unaffected in pot experiments.

This method although it has been used successfully in extracting sporangia from soil as it occurs in the field, *i.e.* in the workhouse grounds at Ormskirk, is more applicable in the case of small quantities of heavily infected soil in the laboratory. It has been tested in conjunction with pot experiments in the following manner. Contaminated soil treated with various chemicals has been put in pots. Small quantites of soil from each pot have been still more heavily contaminated with sporangia, wrapped up in bags made of voile and sunk in the pots for a month, after which the bags were removed and tubers planted in the pots. Sporangia extracted by chloroform from the soil in the bags were examined by the acid fuchsin staining tests and showed little or no deep staining, indicating that they were still capable of causing infection. The results of the pot experiments showed this to be the case.

III. THE EFFECT OF TEMPERATURE ON THE VIABILITY OF THE WINTER SPORANGIUM.

Introduction.

Steam has been found effective in eradicating *Synchytrium endobioticum* from soil by several workers, notably Gimingham and Spinks (2), Kunkel and Orton (5) and by investigators at Rothamsted (9). Except for this fact practically nothing is known of the effect of temperature on the viability of winter sporangia. Information of this type is needed as a basis for pot and field work and is interesting in view of the high resistance of the sporangium to other toxic agents.

Temperature and Viability.

Technique.

Sporangia were obtained fairly free from other organic matter by pressing decaying warts through a finely woven fabric and centrifuging several times. A small quantity of damp sporangial material was introduced into distilled water already heated to the required temperature

in an electric oven. The sporangia were maintained at this temperature for definite lengths of time, then filtered off rapidly and their viability determined by the acid fuchsin test. Counts of three sets of twenty sporangia were made after each treatment and the numbers reacting to acid fuchsin by "faint," "intermediate" and "deep" staining recorded.

As these groups merge into each other it is sometimes a little difficult to ensure prefect discrimination between them and so obtain exact percentages, but the staining reaction affords a means for a general comparison of the viability of sporangia after different treatments.

Dry sporangia subjected to different temperatures were treated in the same way as wet sporangia, except that they were first air dried and then kept dry during treatment.

Temperature and Viability of the Wet Sporangium.

The reactions of winter sporangia after subjection in the wet state to different temperatures for varying lengths of time are shown in Table V, which gives the actual and average numbers of sporangia which produce a faint, intermediate and deep stain with acid fuchsin.

Diagram I, in which time is plotted on the logarithmic scale, represents the average result of each treatment. The central point of each square marks the time and temperature of each reading, and the intensity of shading indicates the proportion of sporangia which gave a faint, intermediate and deep stain with acid fuchsin. A comparison of the relative viability of sporangia after different treatments is thus readily made. The sporangia all stain deeply, that is, they are probably all dead after treatment at 90° C. for 5 minutes, 80° C. for 15 minutes, 70° C. for 1 hour and 60° C. for 8 hours.

Temperature and Viability of the Dry Sporangium.

Observations were made on the effect of high temperature on the staining reaction of dry sporangia to acid fuchsin.

The results showed, Table V, that a very much higher temperature was required to kill sporangia in a dry condition than when wet. Thus while 15 minutes at 80° C. or 1 hour at 70° C. is enough to kill sporangia in the wet state, 1½ hours at 80° C. and 20 hours at 70° C. is not sufficient to kill them when dry. This fact, which is common to most micro-organisms, is of importance in any practical application which may be made of these results. In heating soil for the purpose of killing the sporangia of *Synchytrium endobioticum* it is very important that the soil should be uniformly damp.

Table V.

The Effect of Temperature on the Reaction to Acid Fuchsin of Wet Sporangia.

Temp. ° C.	Time	Staining reaction of Sporangia			Average		
		Faint	Medium	Deep	Faint	Medium	Deep
40	1 hour	20	0	0			
		20	0	0	20	0	0
		19	1	0			
	8 hours	18	0	2			
		17	2	1	18	1	1
		19	1	0			
	16 „	20	0	0			
		20	0	0	19	1	0
		17	3	0			
	24 „	14	4	2			
		13	5	2	15	4	1
		17	3	0			
50	15 mins.	19	0	1			
		18	2	0	19	1	0
		19	1	0			
	30 „	20	0	0			
		20	0	0	19	1	0
		16	4	0			
	1 hour	18	2	0			
		19	1	0	17	3	0
		15	5	0			
	2 hours	18	1	1			
		16	1	3	16	2	2
		15	4	1			
	8 „	15	5	0			
		7	11	2	10	8	2
		9	8	3			
	16 „	3	12	5			
		8	5	7	5	10	5
		4	13	3			
	24 „	4	13	3			
		2	4	14	3	10	7
		1	14	5			
60	5 mins.	16	2	2			
		18	2	0	17	2	1
		19	1	0			
	15 „	8	9	3			
		6	14	0	9	10	1
		12	7	1			
	30 „	10	8	2			
		6	11	3	8	10	2
		7	12	1			
	1 hour	7	10	3			
		1	6	13	4	8	8
		3	7	10			
	2 hours	2	9	9			
		0	4	16	1	7	12
		2	7	11			
	8 „	0	0	20			
		0	0	20	0	0	20
		0	0	20			

Table V (*continued*).

Temp. °C.	Time	Staining reaction of Sporangia					
					Average		
		Faint	Medium	Deep	Faint	Medium	Deep
60	16 hours	0	0	20			
		0	0	20	0	0	20
		0	0	20			
	24 ,,	0	0	20			
		0	0	20	0	0	20
		0	0	20			
70	5 mins.	2	7	11			
		1	3	16	1	5	14
		1	6	13			
	15 ,,	2	5	13			
		0	1	19	1	4	15
		0	5	15			
	30 ,,	0	5	15			
		0	4	16	0	5	15
		0	6	14			
	1 hour	0	0	20			
		0	1	19	0	0	20
		0	0	20			
	2 hours	0	0	20			
		0	1	19	0	0	20
		0	0	20			
80	5 mins.	0	1	19			
		0	1	19	0	1	19
		0	1	19			
	15 ,,	0	1	19			
		0	0	20	0	0	20
		0	0	20			
	30 ,,	0	0	20			
		0	0	20	0	0	20
		0	0	20			
	1 hour	0	0	20			
		0	0	20	0	0	20
		0	0	20			
90	5 mins.	0	0	20			
		0	0	20	0	0	20
		0	0	20			
	15 ,,	0	0	20			
		0	0	20	0	0	20
		0	0	20			
	30 ,,	0	0	20			
		0	0	20	0	0	20
		0	0	20			
	1 hour	0	0	20			
		0	0	20	0	0	20
		0	0	20			
100	5 mins.	0	0	20			
		0	0	20	0	0	20
		0	0	20			
	15 ,,	0	0	20			
		0	0	20	0	0	20
		0	0	20			

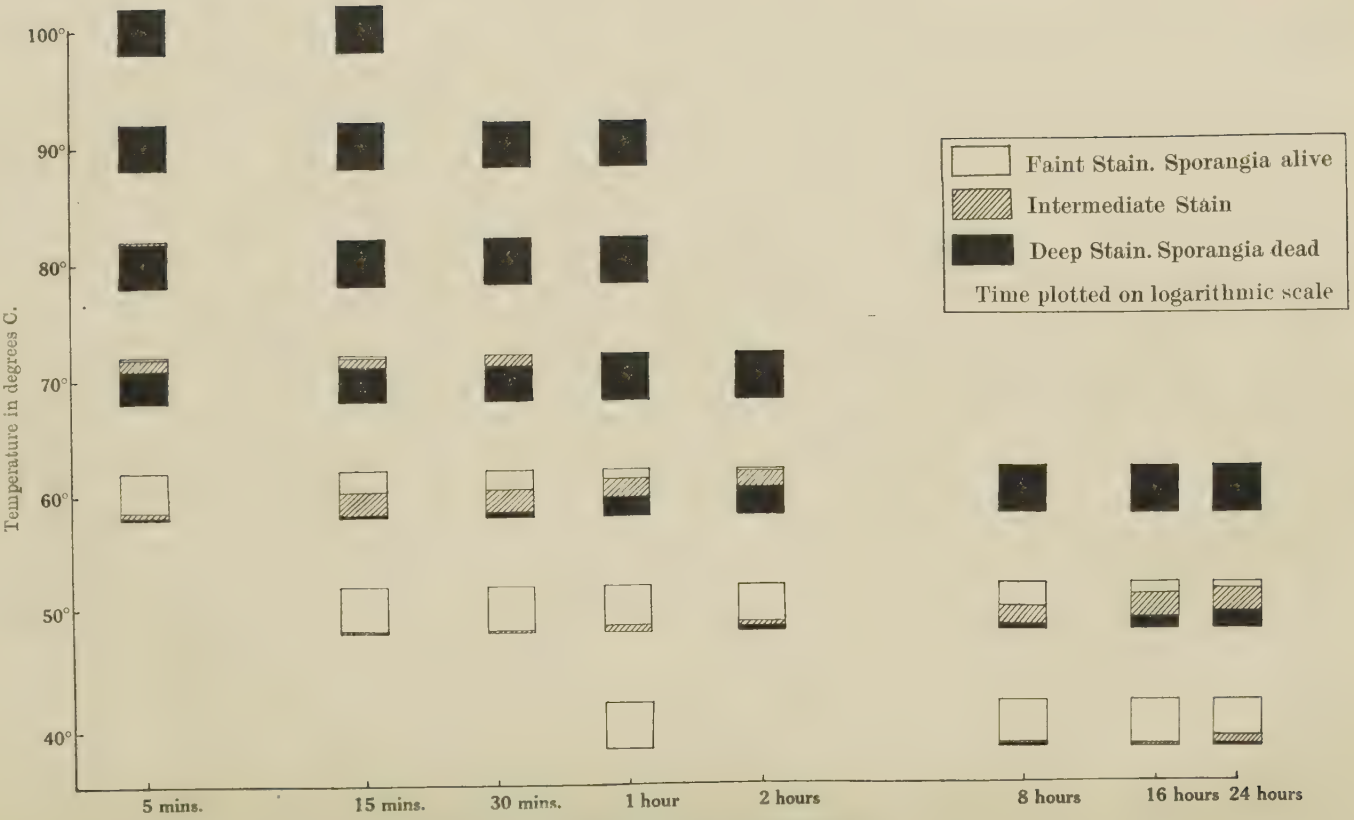

Diagram I. The viability of the winter sporangium.

Table VI.

The Effect of Temperature on the Reaction to Acid Fuchsin of Dry Sporangia.

Temp. ° C.	Time	Staining reaction of Sporangia					
					Average		
		Faint	Medium	Deep	Faint	Medium	Deep
75	½ hour	13	6	1			
		13	5	2	14	5	1
		15	5	0			
75	20 hours	7	9	4			
		12	6	2	8	8	4
		6	8	6			
80	1½ „	19	1	0			
		19	1	0	19	1	0
		20	0	0			
90	5 mins.	18	2	0			
		16	3	1	17	2	1
		18	1	1			
90	15 „	16	4	0			
		16	4	0	17	3	0
		20	0	0			

SUMMARY.

A staining method for testing the viability of the winter sporangia of *Synchytrium endobioticum* is described. The sporangial contents are pressed out into acid fuchsin or after treatment by a strongly alkaline reagent into methylene blue. The staining reactions have been correlated with the results of infection experiments in pots. Sporangia which, like the controls stain faintly, produce a high percentage infection and are therefore alive. Those which stain deeply and rapidly produce no infection and are presumably dead. There is an intermediate group in which some sporangia stain deeply and some are intermediate in reaction. This group tends to give less infection than the controls.

A method whereby sporangia which have been treated in soil may be extracted without affecting their viability is described. The method depends on the difference in specific gravity of sporangia which has been determined as about 1·17 and of soil which is in the region of 2·5. The sporangia are extracted by means of chloroform (sp. gr. 1·5 approx.), which does not affect their viability.

A study of the relation of temperature, time and viability shows that treatment for 5 minutes at 90° C., 15 minutes at 80° C., 1 hour at 70° C. and 8 hours at 60° C. have a similar effect in killing all the sporangia.

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